AWARD NUMBER: W81XWH-14-1-0572

TITLE: Monosodium Luminol for Improving Brain Function in Gulf War Illness

PRINCIPAL INVESTIGATOR: Ashok K. Shetty, Ph.D.

CONTRACTING ORGANIZATION: Texas A&M University System
College Station, TX 77845

REPORT DATE: October 2015

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2015	Annual	30 Sep 2014 - 29 Sep 2015

4. TITLE AND SUBTITLE

Monosodium Luminol for Improving Brain Function in Gulf War Illness

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-14-1-0572

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Ashok K. Shetty, Ph.D.

E-Mail: shetty@medicine.tamhsc.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

THE TEXAS A&M UNIVERSITY
SYSTEM
200 TECHNOLOGY WAY, STE 2079
COLLEGE STATION TX 77845-3424

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The goal of this project is to ascertain whether administration of monosodium luminol-GVT (MSL-GVT, an antioxidant drug from Bach Pharma) in a rat model of Gulf war illness (GWI) would alleviate mood and memory dysfunction and anxiety associated with Gulf war illness (GWI). Specific Aim 1 studies are focused on quantifying the efficacy of different doses of MSL-GVT for suppressing oxidative stress and inflammation and improving neurogenesis in the hippocampus of rats exposed to GWI-related (GWIR) chemicals and stress (GWI-rats). Specific Aim 2 studies are focused on examining whether long-term administration of an apt dose of MSL-GVT would alleviate mood and memory dysfunction and anxiety in GWI-rats, using a battery of behavioral tests. During the past year, a portion of experiments for Specific Aim 1 was performed: (1) Exposure of rats to GWIR-chemicals and moderate stress. (2) Oral administration of different doses of MSL-GVT, 4-months after the exposure. (3) Mood and memory function analyses using a few behavioral tests. (4) Analyses of oxidative stress using biochemical and molecular biological assays. The data collected so far suggest that administration of higher doses of MSL-GVT to GWI-rats improves mood and memory function with decreased concentration of malondialdehyde (a byproduct of oxidative stress) and normalized expression of oxidative stress responsive genes.

15. SUBJECT TERMS

DEET, Gulf war illness, hippocampal neurogenesis, Memory dysfunction, Mood dysfunction, neuroinflammation, oxidative stress, Permethrin, Pyridostigmine bromide, monosodium luminol

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	17	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

1. INTRODUCTION

Gulf war illness (GWI) is a chronic multi-symptom health problem, which afflicts nearly 30% of veterans who served in the Persian Gulf War-1 (PGW-1). Brain dysfunction, typified by memory dysfunction, depression and anxiety, is one of the major health issues in GWI. While the precise etiology of GWI is unknown, several suspected causes have been proposed. Among these, the hypothesis that GWI is linked to a combination of exposures encountered by service personnel during the war has received much attention. First, veterans who were stationed in the battlefield areas believed to have consumed pills of pyridostigmine bromide (PB) during the war. PB was employed as a prophylactic treatment to protect against a possible attack with organophosphate nerve gas agents. Second, preparations for the PGW-1 comprised measures to offset infectious diseases transmitted by insects/ticks in the region. The measures included the use of pesticides for the area protection and insect repellants on the skin and uniforms. The pesticides included the insecticide permethrin (PM) and the insect repellant DEET. Thus, in view of the exposure of service personnel to the above GWI-related (GWIR) chemicals and war related stress, it is hypothesized that the neurological symptoms displayed by a significant number of PGW-1 veterans are due to synergistic interaction of PB with pesticides PM and DEET and/or stress. This chemical exposure hypothesis is also supported by Research advisory committee's (RAC's) report on GWI that the overall prevalence of GWI is greater in veterans who used higher amounts of pesticides than veterans who had limited exposure to pesticides during the PGW-1. Consistent with this theory, studies in our laboratory using rat models have shown that combined exposure to low doses of chemicals PB, PM and DEET with mild/moderate stress for 4 weeks causes dysfunction of the hippocampus, which was typified by impairments in memory and mood function with increased oxidative stress, chronic low-level inflammation and greatly declined neurogenesis. Because all of these changes can adversely affect memory and mood function, drugs capable of suppressing oxidative stress and inflammation and/or increasing neurogenesis have received attention for alleviating cognitive and mood impairments in veterans afflicted with GWI. This project is focused on ascertaining the efficacy of an antioxidant and anti-inflammatory drug monosodium luminol-GVT (MSL-GVT from Bach Pharma) for easing memory and mood dysfunction in a rat model of GWI.

2. KEYWORDS

Anxiety
DEET
Gulf war illness
Hippocampal neurogenesis
Memory dysfunction
Mood dysfunction
Neuroinflammation
Oxidative stress
Permethrin
Pyridostigmine bromide
Monosodium luminol GVT

3. ACCOMPLISHMENTS

3.1. Major Goals:

The major goal of this project is to examine the efficacy of monosodium luminol-GVT (MSL-GVT from Bach Pharma) for alleviating mood and memory dysfunction in a rat model of GWI. The chosen animal model of GWI has been well characterized, simulates the various exposures likely encountered by the veterans during the PGW-1, and reliably induces cognitive and mood dysfunction in association with increased oxidative stress, low-level chronic

inflammation and declined neurogenesis in the hippocampus. Studies in Specific Aim 1 are focused on quantifying the efficacy of oral administration of different doses of MSL-GVT (via oral gavage) for suppressing oxidative stress and inflammation, and stimulating the proliferation of hippocampal neural stem cells (NSCs) and increasing the extent of net neurogenesis in rats exposed to GWI-related (GWIR)-chemicals and moderate levels of stress four months earlier (GWI-rats). The goal is to identify an optimal dose of MSL-GVT that greatly suppresses inflammation and oxidative stress and enhances hippocampal neurogenesis in rats exposed to GWIR-chemicals and moderate levels of stress. Studies in Specific Aim 2 are focused on determining whether oral administration of an apt dose of MSL-GVT for prolonged periods is efficient for alleviating mood and memory dysfunction and anxiety-like behavior in GWI-rats, using a battery of behavioral tests.

3.2. Studies Accomplished During the Past Year:

3.2.1. Specific activities:

The following narrative describes studies accomplished so far for Specific Aim 1 (Task 1). The experiments in this aim comprises 5 major groups of rats:

Group 1: GWI-rats receiving MSL-GVT at 40mg/Kg b.w.
Group 2: GWI-rats receiving MSL-GVT at 80mg/Kg b.w.
Group 3: GWI-rats receiving MSL-GVT at 160mg/Kg b.w.
Group 4: GWI-rats receiving vehicle (VEH)
Group 5: Age-matched naïve control rats

3.2.1.1. Animal numbers, survival and tissue harvesting:

(1) A total of 115 rats have been purchased so far in 3 different cohorts.

(a) The first cohort comprised 31 animals at the start of experiments. From these, 29 animals reached the endpoint of experiments; two animals were found dead during the four-months waiting period between the exposure of animals (to Gulf war illness related (GWIR) chemicals and 15 minutes of restraint stress for 28 days) and the commencement of MSL-GVT treatment. The brain tissues from 29 animals have been harvested for biochemical and molecular biological studies, which belong to the following groups:

(1) GWI-MSL 40mg/Kg, n=6 (2) GWI-MSL 80mg/Kg, n=6
(3) GWI-MSL 160mg/Kg, n=5 (4) GWI-vehicle (VEH), n=6
(5) Naive control, n=6

(b) The second cohort comprised 42 animals at the start of experiments. From these, 39 animals reached the endpoint of experiments. Two animals were found dead during the four-months waiting period between the exposure of animals (to GWIR chemicals and stress and the commencement of MSL-GVT treatment. An additional animal was euthanized in this period because it developed uncontrolled seizures, typified by continuous Stage-V seizures (bilateral forelimb clonus with rearing and falling). The brain tissues from 39 animals have been harvested for immunohistochemical studies, which belong to the following groups:

(1) GWI-MSL 40mg/Kg, n=8 (2) GWI-MSL 80mg/Kg, n=8
(3) GWI-MSL 160mg/Kg, n=8 (4) GWI-VEH, n=8
(5) Naive control, n=7

(c) The third cohort comprised 42 animals at the start of experiments. The experiments are still ongoing for these animals. Out of these, 34 animals have been exposed to GWIR-chemicals and stress and 8 animals are maintained as control group. Animals exposed to GWIR-chemicals and stress will be assigned to VEH or MSL-GVT treatment groups once they complete 4-months of waiting period.

3.2.1.2. Time-line of various procedures for animals in cohorts 1 and 2:

(i) Exposure period to GWIR-chemicals and stress:	28 days (daily)
(ii) Survival period between exposure and treatment:	4 months
(iii) MSL-GVT or VEH treatment period:	8 weeks (5 times/week)
(iv) 5'-bromodeoxyuridine (BrdU) injection period:	5 days (in the 3rd week of treatment)
(iv) Cognitive and mood function tests	Started from 5th week of treatment
(v) Euthanasia and Tissue harvesting:	After 8 weeks of treatment

3.2.1.3. Brief description of procedures performed so far:

(a) Exposure of animals to GWIR-chemicals and stress (Subtask 1a of Task 1): Animals were exposed daily to the following chemicals for 28 days: Pyridostigmine bromide (PB) at 2 mg/kg/day (via oral gavage), DEET at 60 mg/kg/day (via dermal application) and Permethrin at 0.2 mg/kg/day (via dermal application). In addition, animals were subjected daily to 15 minutes of restraint stress using rat restrainers during the above 28-day period.

(b) Survival period between exposure and treatment: Following the exposure to GWIR-chemicals and stress, animals were maintained in the vivarium for four months in regular cages (two per cage) with ad libitum access to food and water.

(c) Administration of MSL-GVT or VEH (Subtask 1b of Task 1): Treatment was given for 8 weeks (5 times/week) via oral gavage, commencing in the 5th month after exposure to GWIR-chemicals and stress. The doses of MSL-GVT employed were 40 mg/Kg, 80 mg/Kg and 160 mg/Kg.

(d) BrdU injections: Subgroups of rats from all groups received BrdU injections in the 3rd week of drug/vehicle treatment daily for 5 days at a dose of 100 mg/Kg/day.

(e) Behavioral tests for assessing cognitive and mood function: We examined animals in all groups through stress-free behavioral tests: Pattern Separation Test (PST) for assessing cognitive function and Sucrose Preference Test (SPT) for assessing mood function.

(f) Euthanasia and tissue harvesting: Animals belonging to cohort 1 were deeply anesthetized with isoflurane in a small chamber, until the animal ceased respiration. Deeply anesthetized animals were decapitated following thoracotomy and brain tissues were dissected rapidly for biochemical and molecular biological studies. Animals belonging to cohort 2 were first deeply anesthetized with isoflurane and then perfused through the heart with 4% paraformaldehyde solution. Fixed tissues were harvested for histological studies.

(g) Analyses of oxidative stress:

The hippocampal tissues obtained from animals belonging to cohort #1 were used for the following measurements:

(i) Analyses of the expression of oxidative stress response and antioxidant genes using "The Rat Oxidative Stress Response PCR Array" from Qiagen: We analyzed the expression of 84 key genes involved in oxidative stress response and antioxidant activity in the hippocampus of animals belonging to different groups using quantitative real time PCR (qRT-PCR), to ascertain the effects of MSL-GVT treatment on oxidative stress.

(ii) Measurement of lipid peroxidation through quantification of malondialdehyde: Lipid peroxides are unstable indicators of oxidative stress in cells that decompose to form more complex and reactive compounds such as malondialdehyde (MDA), a natural bi-product of lipid peroxidation. Hence, we measured MDA in hippocampal tissue extracts from different groups using TBARS Assay Kit.

(iii) Quantification of 3-nitrotyrosine: Increased modification of tyrosine residues in proteins to 3-nitrotyrosine by peroxynitrite or other potential nitrating agents is seen in tissues

subjected to oxidative stress. Hence, we quantified 3-nitrotyrosine in hippocampal tissue extracts from different groups, using the nitrotyrosine ELISA Kit.

(h) Analyses of inflammation:

The hippocampal tissues obtained from animals belonging to cohort #1 were also used for measurement of the relative levels of inflammatory cytokines in different groups of animals. We employed "The Rat Cytokine Plate Array" from Signosis, which facilitated analyses of 16 rat cytokines in a high-throughput manner. The cytokines included: tumor necrosis factor-alpha ((TNF-alpha), interleukin-1 alpha (IL-1alpha), interleukin-1 beta (IL-1beta), vascular endothelial growth factor (VEGF), fibroblast growth factor beta (FGFb), interferon gamma (IFN gamma), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-15 (IL15), leptin, monocyte chemoattractant protein-1 (MCP-1), IFN-gamma-inducible protein 10 (IP-10 or CXCL10), stem cell factor (SCF), Regulated on Activation, Normal T Cell Expressed and Secreted (Rantes), Macrophage inflammatory protein 1 alpha (MIP-1a) and transforming growth factor-beta (TGFbeta).

(i) Immunohistochemical studies: Fixed brain tissues obtained from animals belonging to cohort #2 were processed for cryostat sectioning. Serial sections (every 15th or 20th) through the entire hippocampus are currently being processed for immunohistochemical detection of BrdU+ cells (i.e. newly born cells), doublecortin (DCX, a marker of newly born neurons), glial fibrillary acidic protein, (GFAP, a marker of astrocytes), IBA-1 (a marker of all microglia) and ED-1 a marker of activated microglia. These multiple cell types will be quantified using stereology in the coming year (second year of the project).

3.2.2. Progress details:

Dose-response studies conducted so far using MSL-GVT in GWI rats suggest the following findings:

(a) MSL-GVT treatment at higher doses improves cognitive function in GWI rats:

We examined the cognitive ability of GWI rats belonging to different groups (n=11-14/group) through a pattern separation test (PST)**.** Pattern separation function reflects proficiency for discriminating analogous experiences through storage of similar representations in a non-overlapping manner *(Leutgeb et al., Science, 315: 961-966, 2007; Yassa and Stark, Trends Neurosci, 34: 515-525)*. In this test, each rat successively explored two different sets of identical objects (object types 1 and 2) placed on distinct types of floor patterns (Patterns 1 and 2 [P1 and P2]) for 5 minutes each in the two acquisition trials (separated by 30 minutes). Thirty minutes later, in the testing phase (Trial-3), each rat explored an object from trial 2 (which is now a familiar object) and an object from Trial-1 (which is now a novel object) placed on the floor pattern employed in trial 2 (P2).

(i) Excellent pattern separation ability (i.e. ability to distinguish between similar experiences) in naïve rats was revealed by a greater exploration of the object from trial 1 (i.e. novel object on pattern 2 [NO on P2]) than the object from trial 2 (i.e. familiar object on pattern 2 [FO on P2], $p<0.0001$, Fig. 1).

(ii) GWI rats that received VEH (GWI+VEH) showed no preference for the NO on P2, as they spent nearly similar amounts of time with novel and familiar objects on P2 (Fig. 1), implying loss of ability for pattern separation. Previous studies have showed that this task requires normal levels of dentate neurogenesis *(Jain et al., PLoS One, 7: e46340, 2012; McAvoy et al., Front Syst Neurosci, 9:120. eCollection 2015; Oomen et al., Wiley Interdiscip Rev Cogn Sci. 5: 573-587, 2014).* However, it remains to be examined whether these rats display decreased levels of dentate neurogenesis.

(iii) GWI rats that received lower doses of MSL-GVT (40mg/Kg) remained impaired, which was evidenced by their greater exploration of the object from trial 2 (FO on P2]) than the object from trial 1 (NO on P2, $p<0.05$, Fig. 1), suggesting that low dose of MSL-GVT is not efficacious for reversing pattern separation dysfunction. GWI rats that received moderate doses of MSL-GVT (80 mg/Kg) also remained impaired, as they spent similar amounts of time with novel and familiar objects on P2 ($p>0.05$, Fig. 1). In contrast, GWI rats that received higher doses of MSL-GVT (160 mg/Kg) displayed ability for pattern separation. This was revealed by

their greater exploration of the object from trial 1 (NO on P2) than the object from trial 2 (FO on P2), $p<0.05$, Fig. 1). **Taken together, this study suggested that cognitive impairment pertaining to pattern separation could be reversed through oral administration of relatively higher doses of MSL-GVT in GWI rats.** It remains to be examined however whether this cognitive improvement is related to increased levels of dentate neurogenesis in these rats, in comparison to GWI rats that received VEH during the same period. This relationship will be examined quantitatively in the coming year of this project. In addition, studies in Aim 2 will be examining the effects of this dose of MSL-GVT on multiple other cognitive and memory tests using a battery of behavioral tests, which are planned for the 3rd year of this project.

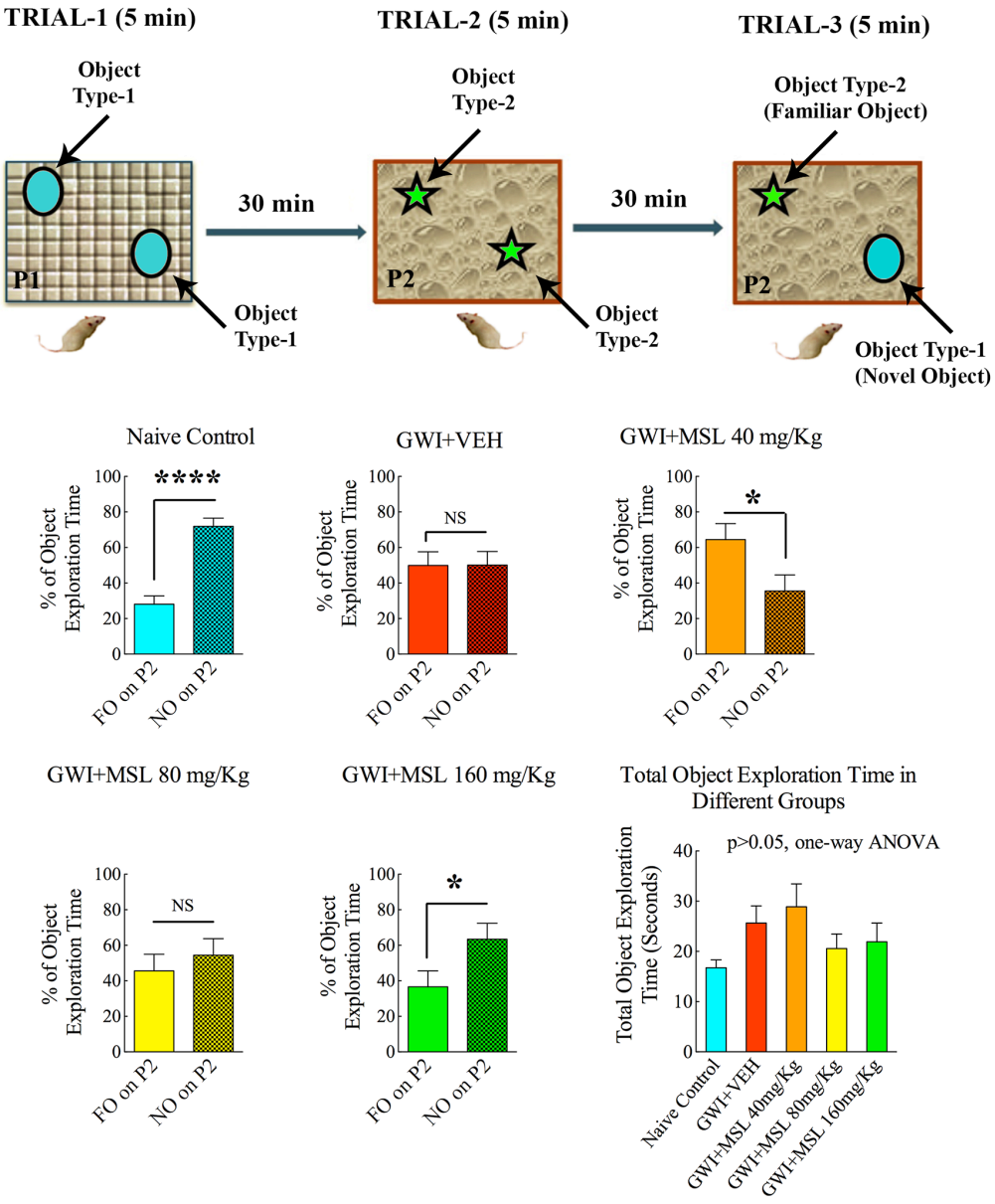

Figure 1 - Results of Pattern Separation Test (PST). Top panel illustrates the sequence of trials, duration of trials, intervals between trials, examples of object types and floor patterns involved in this test. The first five bar charts compare percentages of object exploration time spent with the familiar object on pattern 2 (FO on P2) and the novel object on pattern 2 (NO of P2) in different animal groups (n=11-14/group). Cyan, Naive control group; red, GWI+VEH group; orange, GWI+MSL 40 mg/Kg group; yellow, GWI+MSL 80 mg/Kg group; green, GWI+MSL 160 mg/Kg group. The bar chart on lower right corner compares the total object exploration time between groups in Trial-3. One-way ANOVA analysis did not show differences between groups, implying that the specificity of the novel object exploration time (NO on P2) was not influenced by differences in the total object exploration time.

(b) MSL-GVT treatment at moderate to higher doses improves mood function in GWI rats:

We examined mood function (or the extent of depressive-like behavior) in GWI rats belonging to different groups (n=11-14/group) through a Sucrose Preference Test (SPT), which is a stress free test measuring anhedonia (i.e. inability to feel pleasure, a measure of depression). This test comprised four days of monitoring. On day 1, rats were housed individually and given free access to two identical bottles containing 1% sucrose solution. Rats were trained to adapt to sucrose solution for 24 hours. On day 2, one bottle was replaced with a new bottle containing regular water for 24 hours. On day 3, rats were deprived of water and food for 23 hours, and then on day 4, rats were given free access to two bottles: one containing 100 ml of sucrose solution and another containing 100 ml of regular water. An hour later, the consumed volume in both bottles was recorded.

(i) Naïve control rats clearly showed preference for drinking sucrose-containing water over regular water (Fig. 2).

(ii) GWI rats that received VEH did not exhibit such preference as they consumed normal and sucrose-containing water in equal proportions (Fig. 2), implying the presence of anhedonia in GWI rats.

(ii) GWI rats that received lower doses of MSL-GVT (40mg/Kg) also remained impaired, as they consumed sucrose-containing water and regular water in almost equal proportions (Fig.2), suggesting that lower dose of MSL-GVT does not have positive effect on mood function in GWI rats.

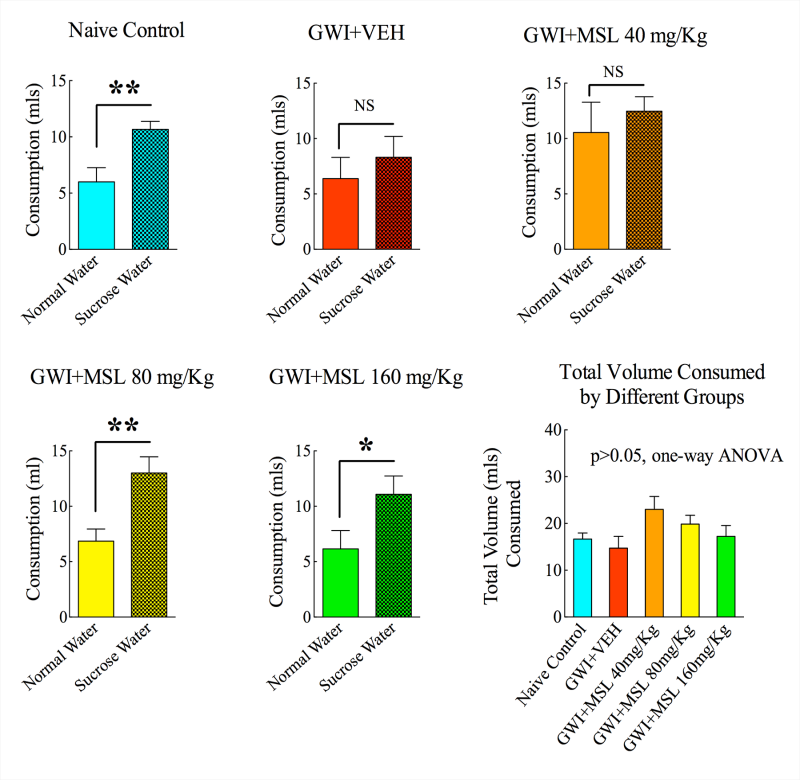

Figure 2 - Results of Sucrose Preference Test (SPT). The first five bar charts compare the consumption of normal water and sucrose containing water in different animal groups (n=13-14/group in al GWI groups, n=6 in naive control group). Cyan, Naive control group; red, GWI+VEH group; orange, GWI+MSL 40 mg/Kg group; yellow, GWI+MSL 80 mg/Kg group; green, GWI+MSL 160 mg/Kg group. The bar chart on lower right corner compares the total volume (normal water + sucrose-containing water) consumed between groups. One-way ANOVA analysis did not show differences between groups, implying that the preference for drinking sucrose-containing water observed in naive control group and MSL-GVT 80 mg/Kg and 160 mg/Kg groups was not influenced by differences in the overall consumption of water during the testing period.

(iv) GWI rats that received moderate and higher doses of MSL-GVT (80 or 160 mg/Kg) exhibited a clear preference for drinking sucrose-containing water over regular water (Fig. 2). Calculation of sucrose preference rate using the formula, sucrose consumption/(water consumption + sucrose consumption) × 100% also showed similar results (data not illustrated).

Thus, the results suggest that mood impairment particularly anhedonia could be reversed with oral administration of moderate to higher doses of MSL-GVT in GWI rats. It is however remains to be examined whether improved mood with MSL-GVT treatment is related to increased levels of dentate neurogenesis in these rats, in comparison to GWI rats that received VEH during the same period. This relationship will be examined quantitatively in the

coming year of this project. In addition, studies in Aim 2 will be examining the effects of MSL-GVT on several mood function tests, which are planned for the 3rd year of this project.

(c) MSL-GVT treatment at higher doses modulates oxidative stress in the hippocampus of GWI rats:

(1) ***Expression of oxidative stress response and antioxidant genes****:* We analyzed the expression of 84 key genes involved in oxidative stress response and antioxidant activity in the hippocampus of animals belonging to different groups using quantitative real time PCR (qRT-PCR). Among 84 genes related to oxidative stress response examined in this experiment, GWI rats receiving VEH exhibited increased expression of 24 genes, in comparison to age-matched naive control animals (Figs. 3 and 4), implying the presence of significant oxidative stress in the hippocampus of GWI rats. Among these 24 genes, the expression of 4 genes was completely normalized by MSL-GVT treatment (Fig. 3). This was evidenced through statistics (one-way ANOVA with Newman-Keuls multiple comparison test), which showed that GWI rats receiving MSL-GVT exhibited reduced expression of these genes, in comparison to GWI rats receiving VEH. The genes comprise the following:

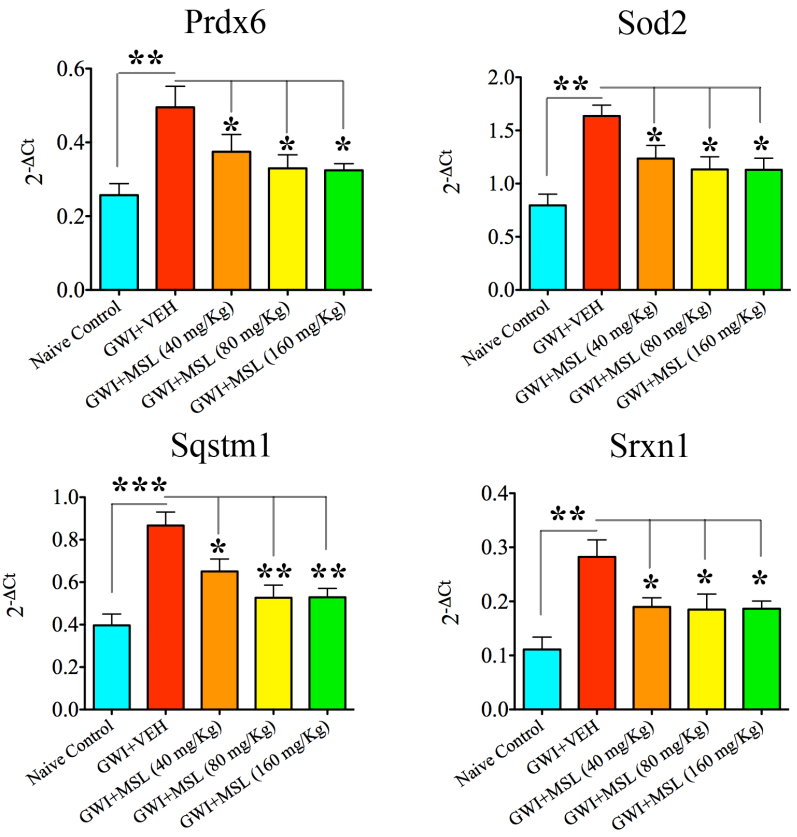

Figure 3: MSL-GVT treatment normalizes the expression of oxidative stress response genes Prdx6, Sod2, Sqstm1 and Srxn1 in GWI rats.

(1) **Prdx6:** This gene encodes peroxiredoxin-6 protein. It is a member of the peroxiredoxin family of antioxidant enzymes (thiol-specific antioxidant protein family). It is involved in redox regulation of the cell, as it can reduce hydrogen peroxide and short chain organic, fatty acid, and phospholipid hydroperoxides. It is also believed to play a role in the regulation of phospholipid turnover as well as in protection against oxidative injury.

(2) **Sod2:** This gene encodes mitochondrial superoxide dismutase 2 protein. It is also known as manganese-dependent superoxide dismutase (MnSOD). Sod2 protein forms a homotetramer and binds one manganese ion per subunit. This protein binds to the superoxide byproducts of oxidative phosphorylation and converts them to hydrogen peroxide and diatomic

oxygen, which facilitates SOD2 to clear mitochondrial reaction oxygen species (ROS) and thereby provides protection against cell death.

(3) **Sqstm1**: This gene encodes sequestosome 1 (or p62) protein. This is a multifunctional protein that binds ubiquitin and regulates activation of the nuclear factor kappa-B (NF-kB) signaling pathway. The protein functions as a scaffolding/adaptor protein in concert with TNF receptor-associated factor 6 to mediate activation of NF-kB in response to upstream signals. Studies also suggest that this protein is a common component of protein aggregates that are found in protein aggregation diseases affecting the brain (e.g. Parkinson's and Alzheimer's diseases).

(4) **Srxn1:** This gene encodes sulfiredoxin-1 protein. This protein binds to peroxiredoxins and reduces over oxidized peroxiredoxins in the presence of cofactors including magnesium and ATP. Elevated expression of this protein has been associated with different types of malignant tumors. Thus, sulfiredoxin along with peroxiredoxins, play an important role in protecting tissues from oxidative stress.

Furthermore, 20 genes that displayed increased expression in GWI rats were normalized to control levels by higher doses of MSL-GVT treatment (80 or 160 mg/Kg, Fig. 4). Their expression was greater in GWI rats receiving VEH than in naive control animals ($p<0.05$-0.01) but did not differ from expression in GWI rats receiving higher doses of MSL-GVT ($p>0.05$). Yet, as the overall reductions were moderate, their expression in GWI rats receiving MSL-GVT did not differ statistically from GWI rats receiving VEH.
The genes include:

(1) Cat gene encoding catalase protein, which is a key antioxidant enzyme that converts the reactive oxygen species hydrogen peroxide to water and oxygen.

(2) Ctsb gene encoding cathepsin B (also called as amyloid precursor protein secretase). This protein is involved in the proteolytic processing of amyloid precursor protein.

(3) Dhcr24 gene encoding 24-dehydrocholesterol reductase, which is an oxidoreductase involved in cholesterol biosynthesis.

(4) Gsr gene encoding glutathione reductase, which reduces oxidized glutathione disulfide to the sulfhydryl form GSH, which is an important cellular antioxidant.

(5) Gstk1 gene encoding glutathione s-transferase kappa 1, which functions in cellular detoxification.

(6) Gstp1 encoding glutathione s-transferase-1, which plays an important role in detoxification by catalyzing the conjugation of many hydrophobic and electrophilic compounds with reduced glutathione.

(7) Idh1 gene encoding isocitrate dehydrogenase 1, which converts isocitrate to 2-ketoglutarate to produce NADPH necessary for many cellular processes and protection against ROS.

(8) Ncf1 encoding neutrophil cytosolic factor 1 protein, which is a 47 kDa cytosolic subunit of neutrophil NADPH oxidase. This oxidase is a multicomponent enzyme that is activated to produce superoxide anion.

(9-12) Prdx1-4 genes encoding peroxiredoxins 1-4, which are antioxidant enzymes involved in reducing hydrogen peroxide and alkyl hydroperoxides to water and alcohol with the use of reducing equivalents derived from thiol-containing donor molecules.

(13) Prnp gene encoding prion protein, which is a membrane glycosylphosphatidylinositol-anchored glycoprotein that tends to aggregate into rod-like structures.

(14) Ptgs2 gene encoding prostaglandin-endoperoxide synthase (also known as cyclooxygenase) which is a key enzyme in prostaglandin biosynthesis, and acts both as a dioxygenase and as a peroxidase.

(15) Slc38a1 gene encoding solute carrier family 38, member 1 protein, which is an important transporter of glutamine, an intermediate in the detoxification of ammonia and the production of urea.

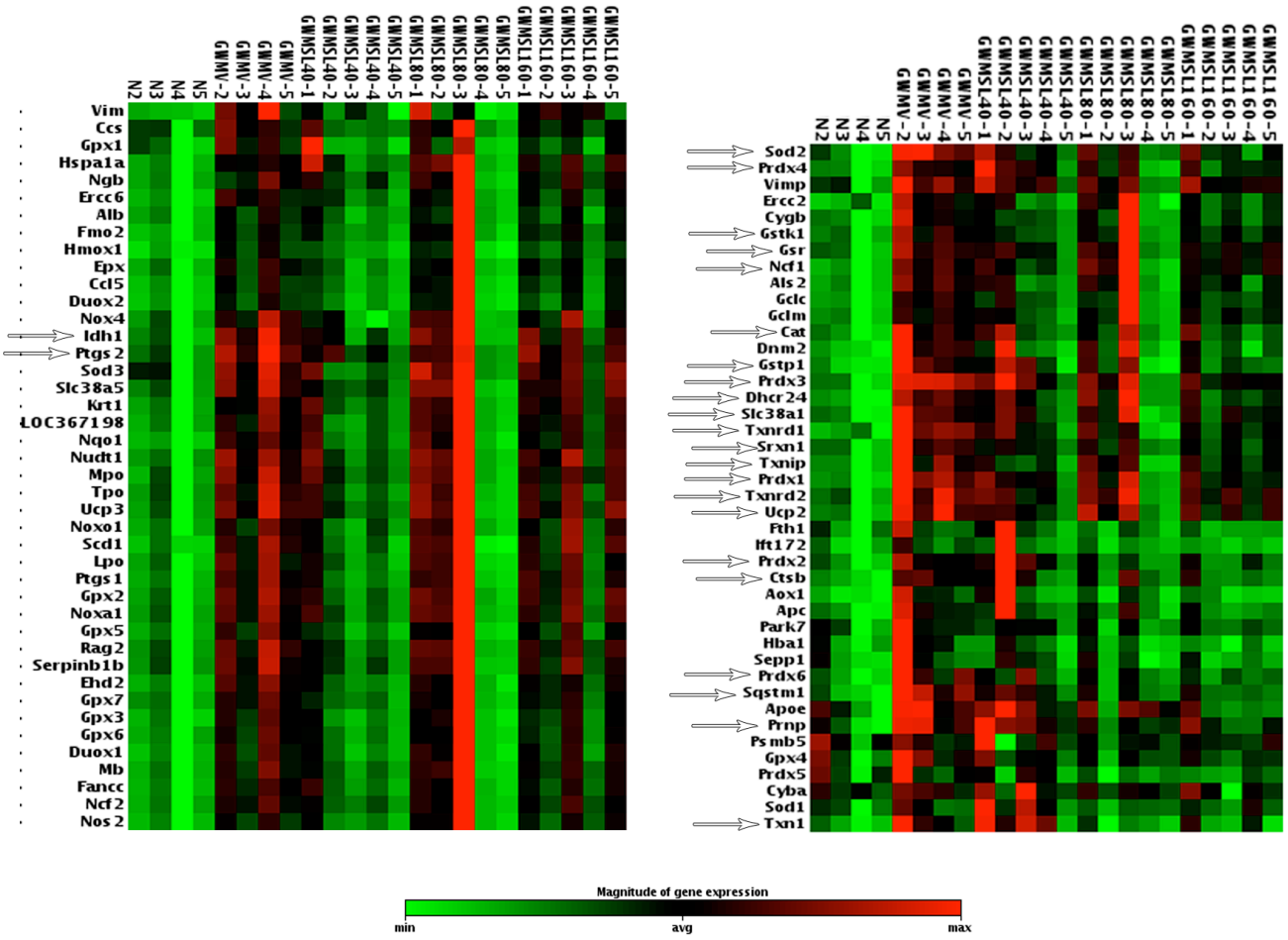

Figure 4: Clustergram showing the expression of oxidative stress response genes in various animal groups. N2-N5, naive control animals (n=4); GWMV2-GWMV5, GWI rats receiving vehicle (n=4); GWMSL40-1 to GWMSL40-5, GWI rats receiving MSL at 40 mg/Kg (n=5); GWMSL80-1 to GWMSL80-5, GWI rats receiving MSL at 80 mg/Kg (n=5); GWMSL160-1 to GWMSL160-5, GWI rats receiving MSL at 160 mg/Kg (n=5). Arrows denote genes, which show upregulation in GWI rats receiving VEH and normalization in GWI rats receiving MSL-GVT, particularly obvious in GWI rats receiving 80 mg/Kg or160mg/Kg doses.

(16) Txn1 gene encoding thioredoxin 1, which participates in various redox reactions through the reversible oxidation of its active center dithiol to a disulfide and catalyzes dithiol-disulfide exchange reactions.

(17) Txnip encoding thioredoxin interacting protein, which is believed to act as an oxidative stress mediator by inhibiting thioredoxin activity or by limiting its bioavailability.

(18) Txnrd1 gene encoding thioredoxin reductase 1, which reduces thioredoxins as well as other substrates, and plays a role in selenium metabolism and protection against oxidative stress.

(19) Txnrd2 gene encoding thioredoxin reductase 2, which is a selenocysteine-containing flavoenzyme that maintains thioredoxins in a reduced state, and thereby plays a key role in regulating the cellular redox environment.

(20) Ucp2 gene encoding uncoupling protein 2 (mitochondrial, proton carrier), which separates oxidative phosphorylation from ATP synthesis with energy dissipated as heat, also referred to as the mitochondrial proton leak. This protein facilitates the transfer of anions from

the inner to the outer mitochondrial membrane and the return transfer of protons from the outer to the inner mitochondrial membrane.

Taken together, our qRT-PCR analyses suggest that MSL-GVT treatment considerably alleviates oxidative stress in GWI rats, which was evidenced through normalization of the expression of multiple genes (that are typically upregulated in conditions such as increased oxidative stress) to levels seen in naive control animals.

Acknowledgments: The summary information on various genes discussed in this document was obtained from GeneCards (human gene database at: http://www.genecards.org), National Center for Biotechnology Information (NCBI at: http://www.ncbi.nlm.nih.gov/gene) and Online Mendelian Inheritance in Man (OMIM at: http://www.omim.org).

(2) ***Levels of malondialdehyde (a measure of lipid peroxidation):*** Lipid peroxides are unstable indicators of oxidative stress in cells that decompose to form more complex and reactive compounds such as malondialdehyde (MDA), a natural bi-product of lipid peroxidation. Hence, we measured MDA in hippocampal tissue extracts from different groups using TBARS Assay Kit. One-way ANOVA analyses showed significant differences between groups ($p<0.01$, $F=5.6$). Post-hoc analyses were done using Newman-Keuls multiple comparison test, which revealed the following findings:

(i) MDA concentration in GWI rats receiving VEH was higher than naive control rats but the difference was not significant statistically.

(ii) GWI rats receiving lower dose of MSL-GVT (40 mg/Kg) displayed higher concentration of MDA than naive control rats ($p<0.05$), GWI rats receiving 80mg/Kg MSL-GVT ($p<0.05$) and GWI rats receiving 160mg/Kg MSL-GVT ($p<0.01$).

(iii) GWI rats receiving higher dose of MSL-GVT (160 mg/Kg) displayed reduced MDA concentration than GWI rats receiving VEH. **Thus, administration of a higher concentration of MSL-GVT (160 mg/Kg) decreases MDA concentration in GWI rats.**

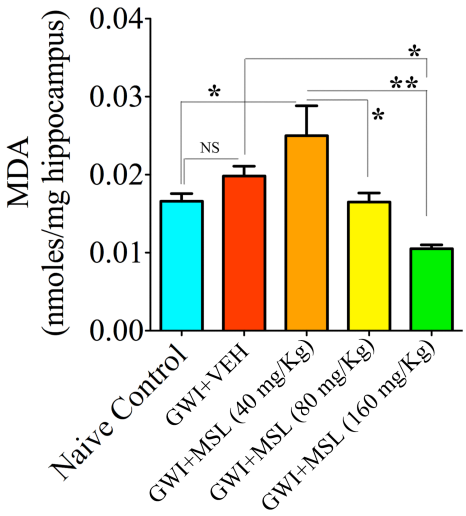

Figure 5 - Results of TBARS assay for MDA: Note that GWI rats receiving 160 mg/Kg MSL-GVT display reduced concentration of MDA than GWI rats receiving VEH.

(3) Concentration of 3-nitrotyrosine*:* Increased modification of tyrosine residues in proteins to 3-nitrotyrosine by peroxynitrite or other potential nitrating agents is seen in tissues subjected to oxidative stress. Hence, we quantified 3-nitrotyrosine in hippocampal tissue extracts from different groups, using the nitrotyrosine ELISA Kit. However, 3-NT levels did not differ between groups (one-way ANOVA, $p>0.05$). Naive control animals and GWI rats that received VEH, and GWI rats that received MSL-GVT exhibited similar levels of 3-NT (Fig. 4). Thus, 3-NT levels are not altered in the hippocampus of GWI rats.

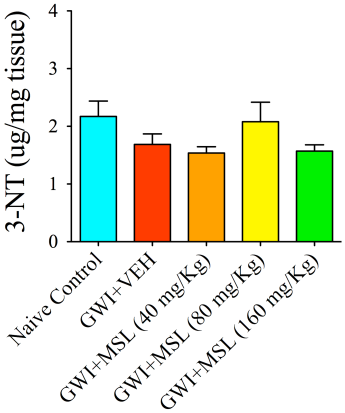

Figure 6 - Results of 3-NT ELISA: Note that GWI rats receiving 160 mg/Kg MSL-GVT display similar levels of 3-NT as other animal groups.

(h) Analyses of inflammation: The hippocampal tissues obtained from animals were used for measurement of the relative levels of inflammatory cytokines in different groups of animals. We employed "The Rat Cytokine Plate Array" from Signosis, which facilitated analyses of 16 rat cytokines in a high-throughput manner. The cytokines included: TNF-alpha, IL-1alpha, IL-1beta,

VEGF, FGFb, IFN gamma, IL-5, IL-6, IL15, leptin, MCP-1, IP-10 (or CXCL10), SCF, Rantes, MIP-1a and TGFbeta.

This study revealed no significant differences in the concentration of these cytokines between naive control animals, GWI rats receiving VEH, and GWI rats receiving different doses of MSL-GVT (see Table 1 below).

Table 1

Cytokine Measured	**Naïve Control Mean ± S.E.M.**	**GWI+VEH Mean ± S.E.M.**	**GWI+MSL 40 mg/Kg Mean ± S.E.M.**	**GWI+MSL 80 mg/Kg Mean ± S.E.M.**	**GWI+MSL 160 mg/Kg Mean ± S.E.M.**	**One-way ANOVA P value**
TNF-alpha	0.15 ± 0.03	0.18 ± 0.02	0.15 ± 0.02	0.19 ± 0.03	0.18 ± 0.02	p>0.05
IL-1 beta	0.32 ±0.07	0.40 ± 0.06	0.38 ± 0.05	0.42 ± 0.06	0.39 ± 0.06	p>0.05
IFN-gamma	0.19± 0.03	0.23± 0.03	0.19± 0.02	0.23± 0.03	0.28± 0.03	p>0.05
Rantes (CCL5)	0.14 ± 0.02	0.18± 0.02	0.15± 0.02	0.17± 0.03	0.18± 0.02	p>0.05
MCP-1	0.12± 0.02	0.14± 0.02	0.09± 0.02	0.13± 0.02	0.10± 0.02	p>0.05
IL-6	0.22± 0.03	0.28± 0.03	0.23± 0.03	0.28± 0.02	0.24± 0.03	p>0.05
IL-1alpha	0.15± 0.03	0.20± 0.03	0.17± 0.02	0.21± 0.03	0.17± 0.01	p>0.05
SCF	0.29± 0.06	0.36± 0.06	0.32± 0.06	0.36± 0.06	0.32± 0.07	p>0.05
***MIP-1 alpha**	**0.05± 0.02**	**0.13± 0.03**	0.06± 0.02	0.07± 0.01	0.08± 0.02	p>0.05
FGF-beta	0.15± 0.03	0.21± 0.03	0.17± 0.03	0.21± 0.03	0.18± 0.03	p>0.05
VEGF	0.18± 0.04	0.22± 0.03	0.19± 0.03	0.22± 0.03	0.21± 0.03	p>0.05
LEPTIN	0.34± 0.09	0.46± 0.09	0.40± 0.08	0.50± 0.09	0.43± 0.11	p>0.05
IL-5	0.17± 0.04	0.21± 0.04	0.19± 0.03	0.22± 0.03	0.19± 0.04	p>0.05
IL-15	0.14± 0.03	0.13± 0.02	0.13± 0.02	0.14± 0.02	0.11± 0.02	p>0.05
IP-10	0.086± 0.02	0.092± 0.02	0.085±0.02	0.103±0.02	0.086± 0.01	p>0.05

*** MIP-1 alpha** (macrophage inflammatory protein-1 alpha, a chemokine)
Naïve control versus GWI+VEH, $p<0.05$ (two-tailed, unpaired t-test)

The only exception is MIP-1alpha, which showed increased expression in GWI rats receiving VEH, in comparison to naive control animals. This protein is produced by macrophages, believed to be involved in inflammation and is typically upregulated in the brain, in conditions such as Alzheimer's disease, multiple sclerosis and hypoxic-ischemic brain injury. Increased expression of this chemokine is believed to enhance inflammation by attracting more leucocytes into the brain parenchyma. Treatment with MSL-GVT reduced the concentration of MIP-1alpha though the decreases were not significant statistically.

3.3. Opportunities for Training and Professional Development:

Nothing to Report

3.4. Dissemination of Results to Communities of Interest:

Nothing to Report

3.5. Plans for the Next Reporting Period:

In the coming year, we will continue and complete Task 1 (Specific Aim 1) experiments.

Specifically, we will perform:

(a) Measurement of ED-1+ activated microglial cells in the hippocampus using stereological cell counting.
(b) Quantification of the area fraction of GFAP+ structural elements in different subfields of the hippocampus using J Image.
(c) Proliferation of neural stem cells (NSCs) in the subgranular zone SGZ using: (i) Ki-67 and Sox-2 dual immunofluorescence and confocal microscopic analyses.
(d) Addition of newly born cells over a period of 5 days via stereological counting of BrdU+ cells in the subgranular zone-granule cell layer (SGZ-GCL) of the dentate gyrus (DG) using serial sections through the hippocampus.
(e) Differentiation of new cells added to the SGZ-GCL into neuron-specific nuclear antigen+ (NeuN+) mature neurons using BrdU and NeuN dual immunofluorescence and confocal microscopic analyses.
(f) Net hippocampal neurogenesis by utilizing data such as the total numbers of BrdU+ cells in the SGZ-GCL and the percentages of BrdU+ newly born cells that differentiate into mature NeuN+ neurons.

In addition, we will commence experiments for Task 2 (Specific Aim 2 experiments), which will involve exposure of animals to GWIR-chemicals and stress followed by MSL-GVT treatment (at extended time-points after exposure).

We will also complete all data analyses for Specific Aim 1 studies, write a manuscript and submit to a peer-reviewed journal for publication.

4. IMPACT:

Data collected from studies conducted so far suggest that oral administration of MSL-GVT at relatively higher doses has considerable promise for alleviating cognitive impairment and mood dysfunction in GWI rats. However, additional studies as proposed in this project are needed for making clear conclusions on the efficacy of MSL-GVT for treating GWI.

5. CHANGES AND PROBLEMS:

(i) Changes in approach:

No changes in approach were required during the past year. None anticipated for the coming year.

(ii) Actual or Anticipated Problems or Delays and Plans to Resolve them:

Nothing to Report

(iii) Changes that had a significant impact on expenditures:

Nothing to Report

(iv) Significant Changes in the use of vertebrate animals or biohazards:

Nothing to Report

(v) Significant Changes in the Care of Vertebrate Animals:

Nothing to Report

6. PRODUCTS:

Publications:

Nothing to Report

7. PARTICIPANTS AND OTHER COLLABORATIVE ORGANIZATIONS

The following research staff members from PI's laboratory were compensated from this grant (for the percentage of effort contributed to this project)

Personnel	Role	Percent Effort
Ashok K. Shetty	Principal Investigator	25%
Bharathi Hattiangady	Assistant Research Professor	25%
Bing Shuai	Senior Research Associate	100%
Geetha Shetty	Research Scientist	17%

Other Collaborators:

Paul Wong (Retired Professor, MD Anderson Cancer Center, Smithville, TX)

Dr. Wong has performed consultation duties to this project during the past year (as described in the original proposal). He has provided advise on handling and preparation of MSL-GVT solution for oral gavage, the dosage and duration of MSL-GVT administration. He was involved actively in discussions (with the PI and research staff) about the procedures and methods for analyses of oxidative stress. He is also overseeing the timely supply of MSL-GVT from Bach Pharma for these studies.

Changes in active other support of the PI or Key Personnel:

There have been no changes in active other support for PI or Key Personnel. One of the research staff working for this project (Geetha Shetty, 17% effort) has left our Institution.

Other Organizations Involved in this Project:

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

None

9. APPENDICES

None